(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 June 2003 (12.06.2003)

PCT

(10) International Publication Number
WO 03/048390 A1

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/IN02/00048

(22) International Filing Date: 20 March 2002 (20.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1222/Del/2001 5 December 2001 (05.12.2001) IN

(71) Applicant and
(72) Inventor: **BHATNAGAR, Rakesh** [IN/IN]; Centre of Biotechnology, Jawaharlal Nehru University, New Delhi - 110 067 (IN).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GUPTA, Pankaj** [IN/IN]; Centre For Biotechnology, Jawaharlal Nehru University, New Delhi 110 067 (IN). **BATRA, Smriti** [IN/IN]; Centre For Biotechnology, Jawaharlal Nehru University, New Delhi 110 067 (IN). **CHAUHAN, Vibha** [IN/IN]; Centre of Biotechnology, Jawaharlal Nehru University, New Delhi - 110 067 (IN). **SINGH, Aparna** [IN/IN]; Centre For Biotechnology, Jawaharlal Nehru University, New Delhi 110 067 (IN). **AHUJA, Nidhi** [IN/IN]; Centre For Biotechnology, Jawaharlal Nehru University, New Delhi 110 067 (IN). **KUMAR, Praveen** [IN/IN]; Centre For Biotechnology, Jawaharlal Nehru University, New Delhi 110 067 (IN).

(74) Agents: **ANAND, Pravin** et al.; Anand & Anand Advocates, B-41, Nizamuddin East, New Delhi 110 013 (IN).

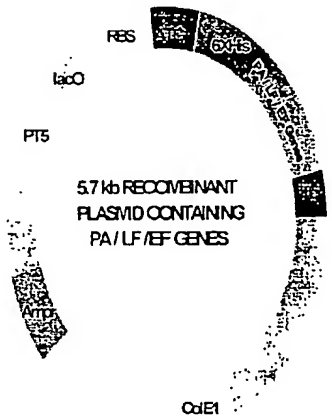
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A PROCESS FOR THE PREPARATION OF A NON-TOXIC ANTHRAX VACCINE



CLONING OF PA, LF and EF IN pQE30 VECTOR

(57) Abstract: Anthrax toxin, comprising of protective antigen (PA), lethal factor (LF) and edema factor (EF) is a major virulent factor of *B.anthracis*. Protective antigen, PA is the main component of all the vaccines against anthrax. The protective efficacy of PA is greatly increased if small quantities of LF or EF are incorporated into the vaccines. An ideal vaccine against anthrax should contain PA, LF and EF together, but this combination would be toxic. Therefore, the biologically inactive mutant preparations of PA, LF and EF may be used together for better immunoprotection. The present invention describes the method for generation of recombinant vaccine against anthrax, comprising of non-toxic, mutant anthrax toxin proteins. The procedure involves site-directed mutagenesis of the native genes of the toxin proteins, the expression and purification of the mutant proteins and finally characterization of these proteins.

WO 03/048390 A1

TITLE OF THE INVENTION

A PROCESS FOR THE PREPARATION OF A NON-TOXIC ANTHRAX VACCINE.

FIELD OF THE INVENTION

This invention relates to a Recombinant DNA construct and a process for the preparation of a non-toxic anthrax vaccine.

BACKGROUND OF THE INVENTION

Anthrax, a zoonotic disease is caused by gram-positive, sporulating bacteria, *Bacillus anthracis*. Humans are accidental hosts through food of animal origin, animal products and contamination of the environment with *Bacillus anthracis (*Brachman P. S., 1970, Annals. N. Y. Acad. Sci. 174, 577-582). Anthrax is one of the oldest known bacterial diseases and occurs in most parts of the world including India. The major virulent factors of *B. anthracis* include poly-D-glutamic acid capsule and a three-component anthrax toxin complex. Anthrax toxin (Leppla S. H., 1991, In Source Book of Bacterial protein toxins, pp277-302.), comprising of protective antigen PA(83 kDa), lethal factor (LF-(90 kDa) and edema factor (EF-(89 kDa) is a major virulent factor of *B. anthracis*. LF/ EF, the catalytic moieties of this complex require PA to enter the cell cytosol. PA in combination with LF (called the lethal toxin), causes death in experimental animals (Smith H. and Keppie J., 1954, Nature, 173, 869-870). PA in combination with EF (called the edema toxin) causes edema in the skin of the experimental animals (Stanley J. L. and Smith H., 1961, J. Gen Microbiol., 26, 49-66). PA is the receptor-binding moiety that facilitates the translocation of the catalytic moieties, LF and EF, into the target cells. After translocation into the cell, LF, a metalloprotease causes cleavage of certain Mitogen activated protein kinase kinases (MAPKKs) resulting in inactivation of

signal transduction pathways (Duesbery N. S., et. al., 1998, Science, 280. 734-737). On the other hand, EF. upon entering the cells, gets activated by calmodulin to cause massive increase in intracellular cAMP levels (Leppla S. H., 1982, Proc. Natl. Acad. Sci. USA., 79, 3162-3166).

The first step of the intoxication process is the binding of PA to the cell surface receptors (Bradley K. A., et al, 2001, Nature, 414, 225-229). After binding to the receptors on the cell surface, PA gets nicked by cell surface proteases to yield a 63-kDa fragment (Klimpel R. K., et. al., 1992, Proc. Natl. Acad. Sci. USA., 89, 10277-10281), which oligomerises and binds to LF/ EF (Milne J. C., et. al. , 1994, J. Biol. Chem. , 269, 20607-20612). Binding of LF/ EF is competitive. The whole complex then undergoes receptor-mediated endocytosis. Acidification of the endosome (Friedlander A. M., 1986, J. Biol. Chem.. 261, 7123-7126) results in the insertion of the PA-oligomer into the endosomal membrane to form pores (Milne J. C. and Collier R. J., 1993, Mol. Microbiol., 10, 647-653) through which LF/ EF are translocated into the cell cytosol.

PA has four domains that are organized primarily into antiparallel-beta sheets with only a few short helices of less than four turns (Petosa C., et. al., 1997, Nature, 385, 833-838). Domain 1 is responsible for binding to LF/EF during the anthrax intoxication process. Domain 2 is dominated by a beta barrel and plays a role in membrane insertion and translocation. Domain 3 is the smallest domain and is important for oligomerization of PA and possibly also in the binding of PA to LF/EF. Domain 4 is the receptor- binding domain.

Crystal structure of LF, determined recently, shows that LF has 4 domains (Pannifer A. D., et al, 2001, Nature, 414, 229-233). Domain 1 is involved in binding to PA. This domain has significant homology to

the N-terminal 1-250 residues of EF. In fact, most of the residues in this region are absolutely conserved.

Of all the three toxin proteins, PA is the most immunogenic and is an essential component of the vaccine against anthrax (Gladstone G. P., 1946, Br. J. Exp. Pathol, 27, 349-418). It has been observed that the protective efficacy of PA is greatly increased if small quantities of LF or EF are incorporated into the vaccine (Pezard et. al., 1995, Infect. Immun., 63, 1369-1372). However, this also happens to be the primary reason of toxigenicity and reactogenicity of the vaccines. Anthrax toxin (Leppla S. H., 1991, In.Source Book of Bacterial protein toxins, pp277-302.), comprising of protective antigen (PA), lethal factor (LF) and edema factor (EF) is a major virulent factor of *B. anthracis*.

The currently used anthrax vaccine is derived from a non-capsulated, avirulent strain of the bacterium known as Sterne's strain (Sterne M., 1939, J. Vet. Sci. Anim. Ind, 13, 307-312). In Russia and China, the live spore vaccines based on Sterne strain are used. In UK the vaccine is alum precipitated culture filtrate of the Sterne strain while the US vaccine consists of an alhydrogel-adsorbed cell free culture filterates of a non-capsulating, non proteolytic derived strain V770 isolated from bovine anthrax (Turnbull P. C. B, 1991, Vaccine, 9, 533-539). All these currently used anthrax vaccines, apart from being crude have undefined composition. They are reactogenic and do not provide protection against all natural strains of *B. anthracis*.

US patent 2,017,606 describes the preparation of anthrax antigen by growing the bacilli with a suitable culture medium, separating the bacilli from the culture medium.

US patent 2,151,364 describes a method of producing an anthrax vaccine which comprises preparing the suspension of anthrax spores,

adding to the suspension a sterile solution containing alum.

RU patent 2,115,433 describes the method of production of anthrax vaccine, which comprises of living spores of non-capsulated strain of *B. anthracis* and protective antigen of *B. anthracis*.

WO patent 0002522 describes a method of production of anthrax vaccine using non-toxic protective antigen from *B. anthracis* for use in inducing immune response, which is protective against anthrax.

The drawbacks in the above-mentioned patents are that all of them use *Bacillus anthracis* cultures/spores. *Bacillus anthracis* is an infectious organism and can not be handled without containment facilities. The vaccine prepared is contaminated with other toxic and non-toxic proteins from *Bacillus anthracis* resulting in a number of side effects and reactogenicity.

These vaccines also have a certain degree of residual virulence for certain species of domesticated and laboratory animals. The Sterne strain is toxigenic and is pathogenic at high doses. As a result it is considered unsafe and unsuitable for human use. This vaccine can cause undesirable side effects including necrosis at the site of inoculation.

Therefore there is a need to develop a second-generation anthrax vaccine which does not have side effects and has a well-defined composition.

The object of the present invention is to render the anthrax toxin non-toxic without affecting its immunogenicity, in order to develop a safe and effective anthrax vaccine.

To achieve said object, the present invention provides a recombinant DNA construct comprising an expression vector and a DNA fragment including genes for wild type Protective Antigen (PA) or wild type Lethal Factor (LF) or wild type Edema Factor (EF)

The present invention also provides a recombinant DNA construct comprising:

an expression vector and a DNA fragment including genes for mutant type Protective Antigen (PA) or mutant type Lethal Factor (LF) or mutant type Edema Factor (EF)

Said vector is a prokaryotic vector such as vector is PQE 30 and said expression vector contains T5 promoter and 6X histidine tag.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe202.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Leu203.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Pro205.

The said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Ile207.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residues Pro205,Trp226 and Phe236.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe552.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Ile574.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe552 and Phe554.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Ile562 and Ile574.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Leu566 and Ile574.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe552and Phe554, Ile562, Leu566 and Ile574.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe427.

The DNA fragment is the gene for protective antigen with deletion of residue Asp 425.

The DNA fragment is the gene for protective antigen with deletion of residue Phe 427.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Trp346.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Leu352.

The DNA fragment is the gene for protective antigen with Alanine-substitution at residue Trp346, Met350 and Leu352.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Tyr148.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Tyr149.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Ile151.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Lys153.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Asp187.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Phe190.

The DNA fragment is the gene for lethal factor with Alanine-substitution at residue Asp187, Leu188, Leu189 and Phe190.

The DNA fragment is the gene for edema factor with Alanine-substitution at residue Tyr137.

The DNA fragment is the gene for edema factor with Alanine-substitution at residue Tyr138.

The DNA fragment is the gene for edema factor with Alanine-substitution at residue Ile140.

The DNA fragment is the gene for edema factor with Alanine-substitution at residue Lys142.

The protein encoded by said DNA fragment is expressed in a prokaryotic host. The said prokaryotic host is an *E.coli* strain.

A protein expressed by gene DNA fragment is wild type PA wild type LF, wild type EF and their mutagenised variants.

This invention further discloses a method for producing mutagenised anthrax toxin protein comprising:

- mutagenizing PA LF & EF genes using different mutagenic primers of the kind as herein defined for PCR reaction;
- treating said mutant PCR product along with the native template with a n enzyme to cleave the native template of said PCR product;
- transforming said mutant product in E.coli strain;
- isolating the recombinant construct from transformed E.coli strain and confirming the desired mutation;
- transforming the confirmed mutant construct in appropriate *E. coli* expression strain to express the mutant protein and
- purifying the said expressed mutant protein.

The purification is carried out using Ni-NTA chromatography and / or other chromatographic techniques.

The genes are cloned in PQE expression vector containing T5 promoter and 6X histidine tag.

The mutations were affected in the first domain of PA at residues 202, 203, 205. The mutations were affected in the third domain of PA at residues 552, 574 552+554, 562+574, 566+574, 552+554+562+566+574 resulting in mutant proteins that were defective in oligomerization. The mutations were affected in the second domain of PA at residues 425 & 427 of loop 4 of domain 2. These mutations impaired the translocation-ability of PA The mutations were affected in the second domain of PA at residues 346, 352 and 346+350+352 in loop 3 of domain 2 such that PA becomes biologically inactive. The mutations were affected in the 1st domain of LF at residues 148, 149, 151, 153, 187, 190 and 187+188+189+190 impaired the binding of LF to PA. The mutations were affected in the 1st 250 residues of EF.

An anthrax vaccine comprising an anthrax toxin protein selected from wild type PA or wild type LF or wild type EF.

An anthrax vaccine comprising an anthrax toxin protein selected from mutant type PA or mutant type LF or mutant type EF or a combination thereof.

An anthrax vaccine comprising an anthrax toxin protein selected is a combination of anyone selected from wild type PA or wild type LF or wild type EF with any one or more selected from mutant type PA or mutant type LF or mutant type EF.

A pharmaceutical composition comprising an effective amount of a anthrax toxin protein as claimed by the present invention.

DETAILED DESCRIPTION OF THE INVENTION

An ideal vaccine against anthrax should contain PA, LF, EF together, but at the same time it should be non-toxic and safe. Purified recombinant proteins with defined composition may be used in the vaccine to minimize reactogenicity of the vaccine. Further, these anthrax toxin proteins maybe rendered non-toxic by introducing mutations that affect the biological activity of the proteins without affecting their structure or immmunogenicity. These non-toxic, mutant anthrax toxin proteins may be used together to create a safe, non-reactogenic and effective recombinant vaccine against anthrax. Thus, the prime objective of this invention was to create a process for making a safe and effective, second-generation vaccine against anthrax comprising of non-toxic anthrax toxin proteins that have been produced by site-directed mutagenesis of the different functionally important domains of the toxin proteins.

The inventors of this application have PCR amplified the genes for PA, LF and EF. They have cloned these genes in pQE30 expression vector (Gupta P., et. al., 1998, Infect. Immun., 66, 862-865; Gupta P., et. al., 1999 Protein Expr. Purif. 16, 369-376; Kumar P., et. al. 2001, Infect. Immun., 69, 6532-6536). The vector contains T5 promoter and a 6x-Histidine tag, which allows convenient purification of the recombinant proteins (Fig.1).

Conditions for overexpression of the said genes using the above mentioned recombinant plasmids, from *E. coli* strains have been optimised by the inventors (Chauhan V., et. al., 2001, Biochem. Biophys. Res. Commun., 283, 308-315).

Using the above mentioned recombinant plasmid, inventors of the present process, introduced mutations in the said genes to make the

expressed recombinant proteins defective in their biological function, thereby rendering them non-toxic. The invention involves the expression and purification of the said mutant proteins from *E. coli* strains. It further involves full characterization of the purified mutant proteins to pinpoint the defect that renders them non-toxic.

MUTATIONS INTRODUCED IN PROTECTIVE ANTIGEN AS PART OF THE INVENTION

1. Mutations that make PA defective in binding to LF/ EF. The inventors introduced series of mutations in the 1st domain of PA. Among the mutations introduced, the mutations at residues 202, 203, 205, 207 and 205+226+236 were found to be defective in binding to LF.
2. Mutations that make PA defective in oligomerization. The authors of this invention introduced mutations in the 3rd domain of PA. The mutation at the residues 552, 574, 552+554, 562+574, 566+574, 552+554+562+566+574 resulted in mutant proteins that were defective in oligomerization.
3. Mutations that make PA translocation-defective. Inventors have introduced mutations at residues 425 and 427 of loop 4 of domain 2. These mutations impaired the translocation-ability of PA.
4. Mutations that make PA defective in insertion/translocation. Authors have discovered that when mutations are introduced at the residues 346, 352 and 346+350+352 in loop 3 of domain 2, PA becomes biologically inactive. The mutant proteins were able to bind to the cell-surface receptors, get proteolytically activated to form oligomers and bind to LF. The biological inactivity of these mutant proteins may pertain to a defect in insertion/translocation.

MUTATIONS INTRODUCED IN LETHAL FACTOR AS PART OF THE INVENTION

Mutations that make LF defective in binding to PA. The inventors of this process have introduced mutations in the 1st domain of LF. They found that mutation at residues 148, 149, 151, 153, 187, 190 and 187+188+189+190 impaired the binding of LF to PA.

MUTATIONS INTRODUCED IN EDEMA FACTOR AS PART OF THE INVENTION

Mutations that make EF defective in binding to PA. The inventors of this process have introduced series of mutations in the 1st 250 residues of EF. It was found that mutation at residues 137, 138, 140 and 142 drastically impaired the binding of EF to PA.

After the expression and purification of the mutant proteins the proteins were evaluated for their biological activity.

Inventors have found that the above-mentioned mutants of PA when added along with wild-type LF, were nontoxic to J774A.1 cells. Likewise mutants of LF when added along with wild-type PA were non-toxic to J774A.1 cells. Similarly, mutants of EF when added along with wild-type PA were unable to produce cAMP-toxicity in CHO cells (Table 2).

The purified mutant protein was analyzed for their biological activity by assaying:

- Ability of PA to bind to cell surface receptors,
- Ability of PA to bind to LF or EF,
- Ability of PA to oligomerize,
- Membrane insertion ability of PA oligomer,
- Ability of PA to translocate LF or EF to the cytosol,

- Ability of lethal toxin to kill macrophage cell lines like RAW264.7and J774A.1
- Ability of edema toxin to elongate CHO cells.

IMMUNIZATION STUDIES

Protective antigen, as the name suggests is a highly immunogenic protein. In fact it is a necessary component of the vaccine against anthrax. Immunization with wild-type recombinant PA elicits high anti-PA titers and provides protection against anthrax lethal challenge in guinea pigs. It was further observed that mutant PA was as immunogenic as the wild-type PA and could easily substitute the wild-type PA in vaccine (Singh et. al. 1998, Infect. Immun. 66, 3447-3448). Immunization studies also indicate a significant contribution of LF/EF to immunoprotection. On basis of these results the inventors have developed a recombinant vaccine against anthrax, which comprises of mutants of all the three anthrax toxin components.

The anthrax toxin based recombinant vaccine developed by the inventors has the following advantages:

1. The process described here does not involve handling of *B. anthracis* cultures (at any stage). This process is therefore safe, cost-effective and does not require the sophisticated containment facilities.
2. The vaccine developed by the inventors has well-defined composition and will therefore not have any batch to batch variation.
3. The invention described here utilizes purified mutant anthrax toxin protein. As a result, this second-generation anthrax vaccine will

not be reactogenic and will not cause any side –effects unlike the previous vaccine.

4. Additionally, this invention comprises of non-toxic mutant proteins, which when administered (either alone or in combination) do not cause any toxigenicity or pathogenicity as associated with the currently used vaccine.

5. The invention described here is therefore safe and suitable for animal/human use.

DETAILS OF THE EXPERIMENTAL PROCEDURES

Site-directed mutagenesis of anthrax toxin proteins

To introduce the desired mutations in the anthrax toxin proteins, complementary mutagenic primers were used (refer Table 1) to amplify the wild type anthrax toxin genes (for PA or LF or EF). High fidelity *Pfu* DNA polymerase was used for the PCR reaction. Entire lengths of both the strands of the plasmid DNA were amplified in a linear fashion during several rounds of thermal cycling, generating a mutant plasmid with staggered nicks on the opposite strands (Fig 2). The amplification was checked by agarose gel electrophoresis of the PCR product. The product of the amplification was treated with *Dpn*I that specifically cleaves fully methylated G^{me6} ATC sequences. The digestion reaction was carried out in 20µl reaction volume with 100ng of the amplified product, 2µl of 10X *Dpn*I reaction buffer and 0.1 U of *Dpn*I. After *Dpn*I digestion, *Dpn*I resistant molecules that are rich in desired mutants were recovered by transformation of the DNA into the appropriate *E. coli* strain. The mutations were confirmed by sequencing of the above constructs using Perkin Elmer cycle DNA sequencing kit.

Expression and purification of the mutant anthrax toxin proteins

The confirmed constructs were transformed into *E. coli* expression strains expressing T5 RNA polymerase. Transformed cells were grown in Luria broth medium (LB) containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin, at 37°C, to an OD_{600} of 0.8. Induction was then done with 0.5 mM IPTG and the incubation was continued at 37°C for 3 to 4 hours. Cells were then harvested by centrifugation at 6000 rpm for 10 minutes. The cells then lysed. The protein profile was analysed by SDS-PAGE and western blotting. The mutant PA proteins were purified using Ni-NTA metal-chelate affinity chromatography and other chromatographic techniques (Kumar P., et. al. 2001, Infect. Immun., 69, 6532-6536; Gupta P., et. al., 1998, Infect. Immun., 66, 862-865; Gupta P., et. al., 1999 Protein Expr. Purif. 16, 369-376). The purified mutant proteins were analysed by SDS-PAGE and western blotting and were estimated using Bradford's method. For storage the purified proteins were dialysed against 50 mM HEPES and stored as aliquotes at –70°C.

Cell culture

Macrophage like cell line J774A.1 was maintained in RPMI 1640 medium containing 10% heat inactivated FCS, 25mM HEPES, 100U/ml penicillin and 200µg/ml streptomycin in a humidified 5% CO_2 environment at 37°C.

CHO cells were maintained in EMEM medium containing 10% heat inactivated FCS, 25mM HEPES, 100U/ml penicillin and 200µg/ml streptomycin in a humidified 5% CO_2 environment at 37°C.

To study the biological activity of the wild-type PA or its mutant proteins, varying concentrations of these proteins were added along with LF (1µg/ml) to J774A.1 cells plated in 96-wells plates. Incubation was allowed for 3 hrs. at 37°C and then cell viability (Bhatnagar et. al. 1989,

Infect. Immun., 57, 2107-2114) was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye (Bhatnagar R., et. al., 1999, Cell Signal.,11, 111-116). MTT dissolved in RPMI was added to each well at a final concentration of 0.5mg/ml and incubated for another 45 min at 37°C to allow uptake and oxidation of the dye by viable cells. The medium was replaced by 0.5% (w/v) sodium dodecyl sulphate (SDS), 25mM HCl in 90% isopropyl alcohol and the plate vortexed. The absorption was read at 540nm using microplate reader (BIORAD).

Similarly, to study the biological activity of wild–type LF or its mutant proteins, varying concentrations of these proteins were added along with PA (1μg/ml) to J774A.1 cells plated in 96-wells plates. Incubation was allowed for 3 hrs. at 37°C and then cell viability was determined using MTT dye, as detailed above.

To study the biological activity of wild–type EF or its mutant proteins, varying concentrations of these proteins were added along with PA (1μg/ml) to CHO cells that were plated in 96-wells plates. Incubation was allowed for 3 hrs. at 37°C and then the cells were microscopically examined for elongation. Rise in intracellular cAMP levels of the cells upon toxin treatment was determined (Kumar P., et. al., 2001, Infect. Immun., 69, 6532-6536) with cAMP EIA kit of Amersham Pharmacia.

Further experiments were then done to understand how mutations were affecting the biological activity of the anthrax toxin mutant proteins.

Binding of PA to cell surface receptors

J774A.1 cells were allowed to grow to confluence in 24-well plates before incubating with 1μg/ml of wild-type PA or its mutant

protein at 4°C for 2 hrs. The cells were then washed with cold RPMI, dissolved in SDS lysis buffer and subjected to SDS-PAGE for electroblotting. The blot was developed with anti-PA antibodies to study the binding of wild-type PA or its mutant protein with the cell surface receptors

Proteolytic cleavage of PA and mutant proteins in solution

Wild-type PA and its mutant proteins were tested for susceptibility to cleavage by trypsin. The proteins (1.0 mg/ml) were incubated with 1μg/ml of trypsin for 30 minutes at room temperature in 25 mM HEPES, 1mM $CaCl_2$, 0.5mM EDTA pH 7.5. The digestion reaction was stopped by adding PMSF to a concentration of 1mM. For SDS-PAGE, the samples were boiled in SDS sample buffer for 5 minutes and resolved on 12 % SDS-PAGE.

Binding of PA to LF on the surface of cells

J774A.1 cells were washed twice with RPMI and then incubated with 1μg/ml of wild-type PA or its mutant protein at 4°C for 3 hrs. The cells were then washed with cold RPMI to remove unbound protein. The cells were further incubated with LF (1.0 μg/ml) for 3 hours and then washed with cold RPMI to remove unbound LF. The cells were dissolved in SDS lysis buffer and subjected to SDS-PAGE for electroblotting. The blot was developed with anti-LF antibodies to study the binding of the wild-type PA or its mutant protein to LF.

Oligomerization of PA in solution

PA upon proteolytic cleavage oligomerises to form heptamers. To study the ability of the wild-type PA and its mutant proteins to form oligomers, the proteins (1mg/ml) were digested with trypsin for 30 minutes at 25°C. The samples were brought to pH 5.0 by addition of 1M

Tris pH5.0 to a final concentration of 100mM. and were boiled for 5 minutes in SDS sample buffer (0.0625M Tris-Cl, 1.25% SDS, 2.5% β-mercaptoethanol and 5% glycerol, pH6.8) before loading on a 3-12% gradient gel. Silver staining was done to detect the formation of oligomers.

Binding of LF/EF to cell-surface bound PA.

J774A.1 cells were washed with cold RPMI and then incubated with 1μg/ml of wild-type PA at 4°C for 3 hrs. The cells were washed again with cold RPMI to remove unbound protein. Wild-type LF/EF or the mutant proteins (1.0 μg/ml) were then added and incubation was continued for 3 hours. The cells were then washed with cold RPMI to remove unbound LF/EF. Later, the cells were dissolved in SDS lysis buffer and subjected to SDS-PAGE for electroblotting. The blot was developed with anti-LF/EF antibodies to study the binding of LF/EF to cell-surface bound .

RESIDUE	CHANGE	PRIMERS	DOMAIN	DEFECT
PA mutants:				
Phe202	To alanine	5'CTTTTCATGAATATTAGAAATCCATGCTGAAAG	I	Defective in binding to Lethal factor
Leu203	To alanine	CTTTTCATGAATATTAGAAATCCATGGTGAAGCAAAAGT	I	Defective in binding to Lethal factor
Pro205	To alanine	CTTTTCATGAATATTAGAAATCCATGGTGAAAGAGCAGTTCT	I	Defective in binding to Lethal factor
Ile207	To alanine	TTTGGTTAACCCTTTCTTTTCATGAATATTAGAAATCCATGGT GAAAGAAAAGTTCTTTTATTTTTGACATCAACCGTATATCCTT CTACCTCTAATGAATCAGCGATTCC	I	Defective in binding to Lethal factor
Pro205 +Trp226 +Phe236	To alanine	CTTTTCATGAATATTAGAAATCCATGGTGAAAGAGCAGTTCT and GGATTTCTAATATTCATGAAAAGAAAGGATTAACCAAATATA AATCATCTCCTGAAAAAGCGAGCACGGCTTCTGATCCGTACA GTGATGCCGAAAAGGTT	I	Defective in binding to Lethal factor
Phe552	To alanine	CAAGGGAAAGATATCACCGAATTTGATGCTAATTTCGATC	III	Oligomerization defective

Ile574	To alanine	GAATTAAACGCGTCTAACGCATATACTG	III	Oligomerization defective
Phe552 +Phe554	To alanine	ATTTTGAGATGTTTGTTGATCGGCATTAGCATCAAATTC	III	Oligomerization defective
Ile562 +Ile574	To alanine	CAGTATATGCGTTAGACGCGTTTAATTCCGCTTAACTGATTCT TGGCATTTTGAGATG	III	Oligomerization defective
Leu566+ Ile574	To alanine	ATCAGGCAGCGGAATTAAACGCGTCTAACGCATATACTG	III	Oligomerization defective
Phe552 +Phe554 + Ile562+Leu566+ Ile574	To alanine	CAGTATATGCGTTAGACGCGTTTAATTCCGCTGCCTGATTCTT GGCATTTTGAGATG and ATTTTGAGATGTTTGTTGATCGGCATTAGCATCAAATT	III	Oligomerization defective
Phe427	To alanine	GTAATTGGAGTAGAACTGGCATCGTCTTGTGC	II	Translocation defective
Asp425	Residue deleted	GTAATTGGAGTAGAACTGAAATCTTGTTCATTTAATGCG	II	Translocation defective
Phe427	Residue deleted	GCACAAGACGATAGTTCTACTCCAATTAC	II	Translocation defective
Trp 346	To alanine	CGGTCGCAATTGATCATTCACTATCTCTAGCAGGGGAAAGAA CTGCGGCTGAAACAATG	II	Membrane insertion/ translocation defective

Leu 352	To alanine	CGGTCGCAATTGATCATTCACTATCTCTAGCAGGGGAAAGAA CTTGGGCTGAAACAATGGGTGCAAATACCGCTGAT	II	Membrane insertion/ translocation defective
Trp 346, Met 350 and Leu 352	To alanine	CGGTCGCAATTGATCATTCACTATCTCTAGCAGGGGAAAGAA CTGCGGCTGAAACAGCGGGTGCAAATACCGCTGAT	II	Membrane insertion/ translocation defective

LF mutants:				
Tyr148	To alanine	GTAGAAGGTACCGAAAAGGCACTGAACGTTGCTTAT	I	Defective in binding to Protective Antigen
Tyr149	To alanine	GTAGAAGGTACCGAAAAGGCACTGAACGTTTATGCTGAA	I	Defective in binding to Protective Antigen
Ile151	To alanine	GTAGAAGGTACCGAAAAGGCACTGAACGTTTATGAAGCAGGT	I	Defective in binding to Protective Antigen
Lys153	To alanine	GTAGAAGGTACCGAAAAGGCACTGAACGTTTATGAAATAGGT GCAATA	I	Defective in binding to Protective Antigen
Asp187	To alanine	TGTGGGATGTTCCTTAAGCTGATTAGTAAATAAAAGAGCTTGT	I	Defective in

		TCATCTGA		binding to Protective Antigen
Phe190	To alanine	TGTGGGATGTTCCTTAAGCTGATTAGTAGCTAAAAGATCTTG	I	Defective in binding to Protective Antigen
Asp187, Leu188, Leu189, Phe190	To alanine	TGTGGGATGTTCCTTAAGCTGATTAGTAGCTGCAGCAGCTTGT TCATCTGA	I	Defective in binding to Protective Antigen

EF mutants:			
Tyr137	To alanine	CCTTACTTATGATATCAAGAGAAATCCCC TTT CC AAT TTC AGC ATA TAC TTC TTT ACT TTG TTC AC	Defective in binding to Protective Antigen
Tyr138	To alanine	CCTTACTTATGATATCAAGAGAAATCCCC TTT CC AAT TTC ATA AGCTAC TTC TTT ACT TTG TTC AC	Defective in binding to Protective Antigen
Ile140	To alanine	CCTTACTTATGATATCAAGAGAAATCCCC TTT CCAGCTTC ATA ATATAC TTC TTT ACT TTG TTC AC	Defective in binding to Protective Antigen
Lys142	To alanine	CCTTACTTATGATATCAAGAGAAATCCCC GCT CC AAT TTC ATA ATATAC TTC TTT ACT TTG TTC AC	Defective in binding to Protective Antigen

TABLE 2:CHARACTERISTICS OF MUTANTS

MUTATION IN PA	RECEPTOR BINDING	TRYPSIN NICKING	OLIGOMER FORMATION	LF/EF BINDING	TOXICITY
Phe202Ala	+	+	+	-	-
Leu203Ala	+	+	+	-	-
Pro205Ala	+	+	+	-	-
Ile207Ala	+	+	+	-	-
Pro205Ala + Trp226Ala + Phe236Ala	+	+	+	-	-
Phe552Ala	+	+	-	-	-
Ile574Ala	+	+	-	-	-
Phe552Ala + Phe554Ala	+	+	-	-	-
Ile562Ala + Ile574Ala	+	+	-	-	-
Leu566Ala + Ile574Ala	+	+	--	-	-
Phe552Ala + Phe554Ala+ Ile562Ala + Leu566ala + Ile574Ala	+	+	-	-	-
Phe427Ala	+	+	+	+	-
Asp425del	+	+	+	+	-
Phe427del	+	+	+	+	-
Trp346Ala	+	+	+	+	-
Leu352Ala	+		+	+	-
Trp346Ala+ Met350Ala+ Leu352Ala	+	+	+	+	-

MUTATION IN LF	BINDING TO PA	TOXICITY
Tyr148Ala	-	-
Tyr149Ala	-	-
Ile151Ala	-	-
Lys153Ala	-	-
Asp187Ala	-	-
Phe190Ala	-	-
Asp187Ala+Leu188Ala+ Phe190Leu189Ala	-	-

MUTATION IN EF	BINDING TO PA	TOXICITY
Tyr137Ala	-	-
Tyr138Ala	-	-
Ile140Ala	-	-
Lys142Ala	-	-

We claim:

1. A recombinant DNA construct comprising:
   - an expression vector, and
   - a DNA fragment including genes for wild type Protective Antigen (PA) or wild type Lethal Factor (LF) or wild type Edema Factor (EF)

2. A recombinant DNA construct comprising:
   - an expression vector, and
   - a DNA fragment including genes for mutant type Protective Antigen (PA) or mutant type Lethal Factor (LF) or mutant type Edema Factor (EF)

3. A recombinant DNA construct as claimed in claim 1 or 2 wherein said vector is a prokaryotic vector.

4. A recombinant DNA construct as claimed in claim 3 wherein said prokaryotic vector is PQE 30.

5. A recombinant DNA construct as claimed in claim 4 wherein said expression vector contains T5 promoter and 6X histidine tag.

6. A recombinant DNA construct as claimed in claim 1 or 2 wherein said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe202.

7. A recombinant DNA construct as claimed in claim 1 or 2 wherein said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Leu203.

8. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Pro205.

9. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Ile207.

10. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residues Pro205,Trp226 and Phe236.

11. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe552.

12. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Ile574.

13. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe552 and Phe554.

14. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Ile562 and Ile574.

15. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Leu566 and Ile574.

16. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe552and Phe554, Ile562, Leu566 and Ile574.

17. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Phe427.

18. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with deletion of residue Asp425.

19. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with deletion of residue Phe427.

20. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Trp346.

21. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Leu352.

22. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for protective antigen with Alanine-substitution at residue Trp346, Met350 and Leu352.

23. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Tyr148.

24. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Tyr149.

25. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Ile151.

26. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Lys153.

27. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Asp187.

28. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Phe190.

29. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for lethal factor with Alanine-substitution at residue Asp187, Leu188, Leu189 and Phe190.

30. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for edema factor with Alanine-substitution at residue Tyr137.

31. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for edema factor with Alanine-substitution at residue Tyr138.

32. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for edema factor with Alanine-substitution at residue Ile140.

33. A recombinant DNA construct as claimed in claim 1 or 2 wherein the said DNA fragment is the gene for edema factor with Alanine-substitution at residue Lys142.

34. A recombinant DNA construct as claimed in claim 1 or 2 wherein the protein encoded by said DNA fragment is expressed in a prokaryotic host.

35. A recombinant DNA construct as claimed in claim 34 wherein said prokaryotic host is an *E.coli* strain.

36. A protein expressed by gene DNA fragment is wild type PA wild type LF, wild type EF and their mutagenised variants.

37. A method for producing mutagenised anthrax toxin protein comprising:

- mutagenizing PA LF & EF genes using different complementary mutagenic primers for PCR reaction,
- treating said mutant PCR product along with the native template with an enzyme to cleave the native template of said PCR product,
- transforming said mutant product in E.coli strain
- isolating the recombinant construct from transformed E.coli strain and confirming the desired mutation,
- transforming the confirmed mutant construct in appropriate *E. coli* expression strain to express the mutant protein,
- purifying the said expressed mutant protein.

38. A method as claimed in claim 37 wherein said purification is carried out using Ni-NTA chromatography and / or other chromatographic techniques.

39. A method as claimed in claim 37 wherein said enzyme is DpnI enzyme that specifically cleaves fully methylated G^{me6} ATC sequences

40. A method as claimed in claim 37 wherein said genes are cloned in PQE expression vector containing T5 promoters and 6X hystidine tag.

41. A method as claimed in claim 38 wherein mutations were affected in the first domain of PA at residues 202, 203, 205.

42. A method as claimed in claim 37 wherein mutations were affected in the third domain of PA at residues 552, 574 552+554, 562+574, 566+574, 552+554+562+566+574 resulting in mutant proteins that were defective in oligomerization.

43. A method as claimed in claim 37 wherein mutations were affected in the second domain of PA at residues 425 & 427 of loop 4 of domain 2. These mutations impaired the translocation-ability of PA.

44. A method as claimed in claim 37 wherein mutations were affected in the second domain of PA at residues 346, 352 and 346+350+352 in loop 3 of domain 2 such that PA becomes biologically inactive

45. A method as claimed in claim 37 wherein mutations were affected in the 1st domain of LF at residues 148, 149, 151, 153, 187, 190 and 187+188+189+190 impaired the binding of LF to PA.

46. A method as claimed in claim 37 wherein mutations were affected in the 1st 250 residues of EF.

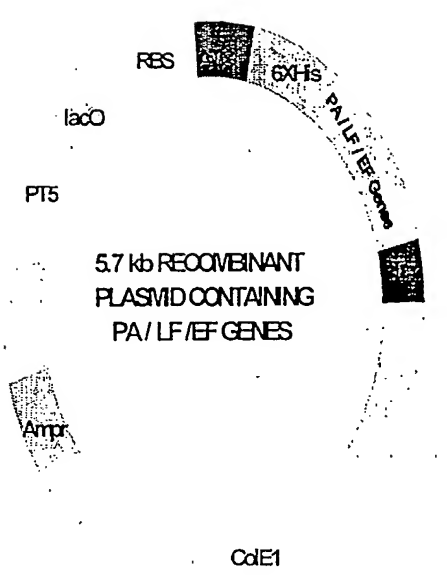
47. An anthrax vaccine comprising an anthrax toxin protein selected from wild type PA or wild type LF or wild type EF.

48. An anthrax vaccine comprising an anthrax toxin protein selected from mutant type PA or mutant type LF or mutant type EF or a combination thereof.

49. An anthrax vaccine comprising an anthrax toxin protein selected is a combination of anyone selected from wild type PA or wild type LF or wild type EF with any one or more selected from mutant type PA or mutant type LF or mutant type EF.

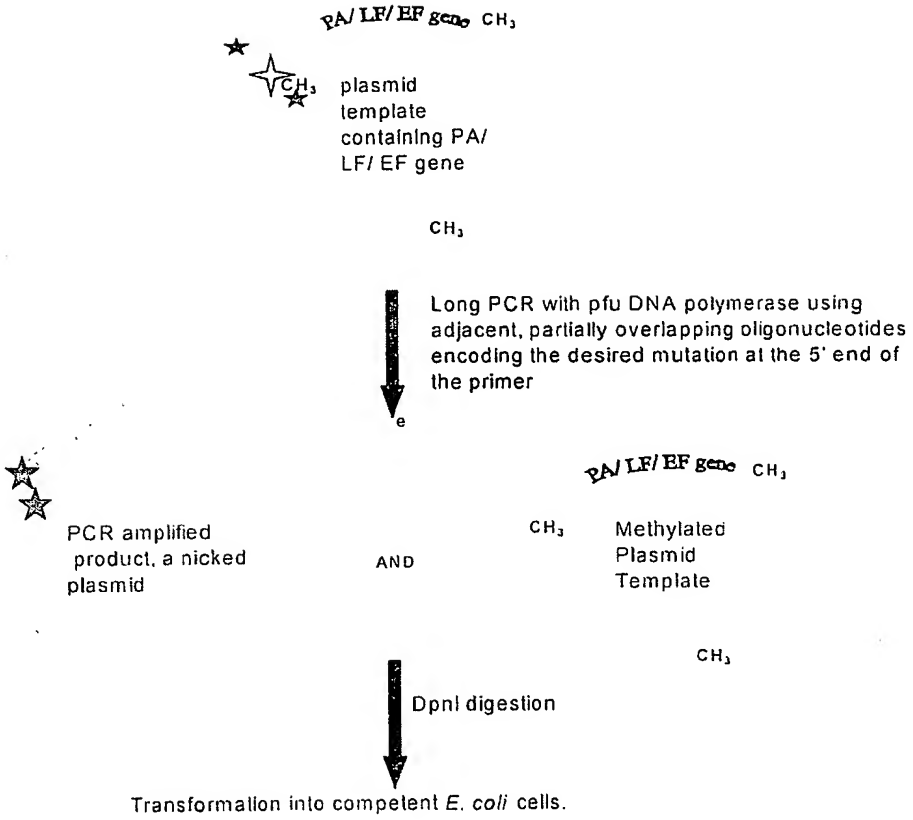
50. A pharmaceutical composition comprising an effective amount of a anthrax toxin protein as claimed in any one of 46- 49.

Figure: 1



CLONING OF PA, LF and EF IN pQE30 VECTOR

Figure:2



Site-Directed Mutagenesis using long PCR

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IN 01/00048

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, PAJ, WPI Data, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XU, R-L., ET AL.: "cloning and sequence analysis of a specific mitochondrial DNA fragment related to wild abortive type cytoplasmic male sterility in rice" ACTA BOTANICA SINICA, vol. 37, no. 7, 1995, pages 501-506, XP008001054 the whole document ---	13
X	DATABASE CHEMICAL ABSTRACTS 'Online! 1994 WEI, G., ET AL.: "cloning and structural analysis of mitochondrial atp6 gene of rice (Oryza sativa L.)" Database accession no. 122:232160 XP002201406 abstract --- -/--	13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2002	23. 07. 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	AKAGI HIROMORI ET AL: "A codominant DNA marker closely linked to the rice nuclear restorer gene, Rf-1, identified with inter-SSR fingerprinting." GENOME, vol. 39, no. 6, 1996, pages 1205-1209, XP002201403 ISSN: 0831-2796 the whole document	22-36
X	LANG NGUYEN T ET AL: "Development of PCR-based markers for thermosensitive genetic male sterility gene tms3(t) in rice (Oryza sativa L.)." HEREDITAS (LUND), vol. 131, no. 2, November 1999 (1999-11), pages 121-127, XP002201404 ISSN: 0018-0661 see Biosis abstract the whole document	22-36
X	-& DATABASE BIOSIS DATABASE 'Online! 1999 LANG NGUYEN T., ET AL.: "Development of PCR-based markers for thermosensitive genetic male sterility gene tms3(t) in rice (Oryza sativa L.)." XP002201407 abstract	22-36
X	DATABASE WPI Section Ch, Week 199819 Derwent Publications Ltd., London, GB; Class C06, AN 1998-210405 XP002201408 & JP 10 057073 A (MITSUI TOATSU CHEM INC), 3 March 1998 (1998-03-03) abstract	29-36
X	REDDY O U K ET AL: "Genetic analysis of temperature-sensitive male sterilty in rice." THEORETICAL AND APPLIED GENETICS, vol. 100, no. 5, March 2000 (2000-03), pages 794-801, XP002201405 ISSN: 0040-5752 figure 3	22-36

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL SEQUENCE LIBRARY 'Online! 14 April 1995 (1995-04-14) NAKAZONO M., ET AL.: "The rps3-rpl16-nad3-rps12 gene cluster in rice mitochondrial DNA is transcribed from alternative promoters - Mitochondrion Oryza sativa genes for ribosomal protein S3, L16, S12 and NADH dehydrogenase subunit 3" XP002188737 cited in the application accession no. D21251 ---	
A	SANE A P ET AL: "RAPD analysis of isolated mitochondrial DNA reveals heterogeneity in elite wild abortive (WA) cytoplasm in rice." THEORETICAL AND APPLIED GENETICS, vol. 95, no. 7, November 1997 (1997-11), pages 1098-1103, XP002188734 ISSN: 0040-5752 page 1099, left-hand column, last paragraph ---	
A	DATABASE WPI Section Ch, Week 199808 Derwent Publications Ltd., London, GB; Class C06, AN 1998-080078 XP002188738 & JP 09 313187 A (MITSUI TOATSU CHEM INC), 9 December 1997 (1997-12-09) abstract ---	
A	WO 00 61812 A (CENTRE NAT RECH SCIENT ;UNIV UTAH (US)) 19 October 2000 (2000-10-19) the whole document ---	
A	WO 98 56948 A (RIDDELL CHRISTINE M ;TULSIERAM LOMAS K (CA); PIONEER HI BRED INT () 17 December 1998 (1998-12-17) examples table 3 ---	
A	WO 98 48611 A (YAN WENGUI) 5 November 1998 (1998-11-05) cited in the application page 12 -page 13 ---	

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IN 01/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DONG N V ET AL: "Molecular mapping of a rice gene conditioning thermosensitive genic male sterility using AFLP, RFLP and SSR techniques." THEORETICAL AND APPLIED GENETICS, vol. 100, no. 5, March 2000 (2000-03), pages 727-734, XP002188736 ISSN: 0040-5752	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN 01/00048

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-12 completely

DNA sequence as characterized by SEQIDs1+3 which is homologous to the rice mitochondrial DNA and being unique to the wild abortive (WA) cytoplasm; oligonucleotide primers as characterized by SEQIDs4+5 used in a PCR assay to distinguish CMS lines of rice from their cognate maintainer lines; a method of using said primers in said PCR assay, also when said CMS lines contain the WA cytoplasm; a method of using said primers in a PCR assay to distinguish WA CMS lines of rice from their cognate maintainer lines wherein an amplification product is obtained when the template DNA is from the CMS line and is not obtained when the template is from the maintainer line; and wherwin the detection of the amplified fragments is by
a) agarose gel electrophoresis
b) detection of the radioactively labeled nucleotide that is incorporated in the PCR amplified product
c) colorimetry, chemiluminescence or measurement of fluorescence
d) PCR-ELISA
e) FRET based detection systems
Furthermore, a method of using multiplex PCR assay of a first primer pair having SEIDs 4+5 in conjunction with a second primer pair which can be derived from any portion of the rice genome outside the region targeted by the first primer pair.

2. Claim : 13 completely

Method of using a CMS specific DNA sequence in a Southern hybridisation assay to distinguish between WA cytoplasmic male sterile lines of rice from their cognate maintainer lines.

3. Claims: 14-21 completely

Method of using co-dominant sequence specific DNA markers like microsatellites or sequence tagged sites (STSs) in a PCR assay for assessing the extent of outcrossing with rogue pollen donors during the multiplication of CMS lines of rice or other crop plants and wherein the DNA is isolated from a single seedling and the genotype is assessed by using agarose or polyacrylamide gels and detection is by methods that use radioactive or non-radioactive labeling; said method wherein the extent of impurities is judged by estimating allele frequencies within the population at the locus which is being assessed, said estimation of allele ferquencies involves separation of fluorescently labeled PCR-amplified fragments.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

4. Claims: 22-28 completely

Method of using co-dominant sequence specific DNA markers like microsatellites or sequence tagged sites (STSs) in a PCR assay for assessing the extent of purity of parental lines of rice hybrids or any economically important crop wherein a two line system of hybrid rice production is followed and wherein the DNA is isolated from a single seedling and the genotype is assessed by using agarose or polyacrylamide gels and detection is by methods that use radioactive or non-radioactive labeling, said method wherein the extent of impurities is judged by estimating allele frequencies within the population at the locus which is being assessed, said estimation of allele ferquencies involves separation of fluorescently labeled PCR-amplified fragments.

5. Claims: 29-36 completely

Method of using co-dominant sequence specific DNA markers like microsatellites or sequence tagged sites (STSs) in a PCR assay for assessing the purity of hybrid seeds (or plants) of rice or any economically valuable crop and either a Three line or Two line system is used for hybrid production and wherein the DNA is isolated from a single seedling and the genotype is assessed by using agarose or polyacrylamide gels and detection is by methods that use radioactive or non-radioactive labeling; said method wherein the DNA is isolated from a population of seedlings and the extent of impurities is judged by estimating allele frequencies within the population at the locus which is being assessed.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IN 01/00048

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
JP 10057073	A	03-03-1998	NONE		
JP 9313187	A	09-12-1997	NONE		
WO 0061812	A	19-10-2000	AU	4225000 A	14-11-2000
			WO	0061812 A1	19-10-2000
			US	6346612 B1	12-02-2002
WO 9856948	A	17-12-1998	CA	2206673 A1	10-12-1998
			AU	8004898 A	30-12-1998
			WO	9856948 A1	17-12-1998
			JP	2002512523 T	23-04-2002
WO 9848611	A	05-11-1998	CN	1187292 A	15-07-1998
			AU	7259598 A	24-11-1998
			WO	9848611 A1	05-11-1998
			CN	1253472 T	17-05-2000
			EP	0982981 A1	08-03-2000
			US	6066779 A	23-05-2000

THIS PAGE LEFT BLANK